



Decreased Expression of the Protein Orhodenticle Homeobox 2 (Otx2) in Individuals With Schizophrenia: A Postmortem Investigation

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Decreased Expression of the Protein Orthodenticle Homeobox 2 (Otx2) in Individuals
with Schizophrenia: A Postmortem Investigation

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Abstract

The purpose of this thesis was to determine if the amount of the protein Orthodenticle homeobox 2 (Otx2) is reduced in individuals with schizophrenia. Otx2 is a transcription factor that plays an essential role in the development and maintenance of a specific class of GABAergic interneurons that express the protein parvalbumin (PV+ GABAergic interneurons). In addition, Otx2 participates in the development and maintenance of perineuronal nets. Perineuronal nets are important extracellular matrix structures that encompass PV+ GABAergic interneurons and facilitate their proper functioning (Beurdeley, 2012). It is speculated that perineuronal net structural abnormalities can lead to impaired PV+ GABAergic interneuron function and these impairments contribute to the manifestation of the condition schizophrenia (Berretta et al., 2015). Because Otx2 is needed for both healthy PV+ GABAergic interneurons and functional perineuronal nets, we postulated that insufficient levels of Otx2 in the amygdala might contribute to impaired GABAergic neurotransmission as exhibited in individuals with schizophrenia. Interestingly, PV+ GABAergic interneurons that require Otx2 do not produce Otx2. Recently, it was shown in mouse models that Otx2 is made in the choroid plexus, released into the cerebral spinal fluid and carried to cortical areas where it becomes available to be used by neurons (Spattazza et al., 2013). Similarly, in human postmortem samples, we observed the presence of Otx2 in the choroid plexus (Pantazopoulos et al., Manuscript in Preparation). Furthermore, we observed a novel phenomenon in human samples; Otx2 was found in vesicles in the cerebral spinal fluid

and in the amygdala samples. We hypothesized that PV+ GABAergic interneurons located in the amygdala obtained Otx2 from the choroid plexus by delivery in vesicles via the cerebral spinal fluid. Based on this observation and building on current scientific understanding of schizophrenia, we proposed that there would be fewer vesicles in the amygdala samples donated by individuals with schizophrenia compared to samples donated by healthy controls. To test this hypothesis, the number of vesicles that contained Otx2 in amygdala samples donated by individuals diagnosed with schizophrenia (n=15) was compared to the number of vesicles that contained Otx2 in amygdala samples donated by healthy control donors (n=15). The results showed that there was not a significant decrease in the number of vesicles that contained Otx2 in amygdala samples donated by individuals with schizophrenia compared to samples donated by healthy controls (p-value > 0.05). However, an enzyme linked immunosorbent assay (ELISA) was performed to measure the amount of Otx2 inside vesicles and a significant decrease in the amount of Otx2 was observed in cerebral spinal fluid samples donated by individuals with schizophrenia (n=11) compared to healthy controls (n=15), (p-value < 0.05). In conclusion, this thesis work honed in on three major ideas. First, the method of delivery of Otx2 to neurons in the amygdala appeared to be via vesicles filled with Otx2, which originated in the choroid plexus. Second, there was not a decrease in the number of vesicles that contained Otx2 in samples from individuals with schizophrenia. Finally, preliminary experiments that measured Otx2 protein concentration inside the vesicles indicated that the amount of Otx2 contained in the vesicles was reduced in samples donated by individuals with schizophrenia compared to healthy controls. These finding

are potential first steps in the development of a bioassay for the molecular characterization of the condition of schizophrenia.

Biographical Sketch

The author's academic interests have been strongly inspired by her parents who lived in Poland during the holocaust. As adults, her parents immigrated to the United States and prospered as scientists. The author studied biology as an undergraduate at Boston University but developed a strong interest in the discipline of psychology. Upon learning about research, which proposed posttraumatic stress disorder could be transmitted through multiple generations, the author became interested in the merger of the disciplines of psychology and biology to develop a more comprehensive understanding of behavioral disorders.

This thesis is dedicated to the memory of Dr. Piotr Kulesza

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Chapter I

Introduction

Schizophrenia is a chronic and debilitating neuropsychiatric condition that has affected individuals throughout history. Although the term schizophrenia was only coined in the 19th century, individuals with “psychotic-like conditions” have been described in various written sources as early as 1,000 B.C. (Evans, Mcgrath, & Milins, 2003; Weinberger, 2010).

For example, writings from the Old Testament indicate that King Saul experienced paranoia, along with visual and auditory hallucinations. Anecdotal accounts of his symptoms have lead researchers to postulate that King Saul was afflicted with a condition akin to schizophrenia (Heinrichs, 2003 & Weinberger, 2010).

Furthermore, individuals with “schizophrenic-like psychosis” were described in Greece during the 10th century B.C. as being mad. It was generally believed that the cause of madness was an infliction by the gods, rather than a psychological disorder with a biological origin. Afflicted individuals were sent to temples in locations such as Aesculapius for various treatments (Hinshaw, 2007).

During the age of Pericleas, Hippocrates (460-377 B.C.) advanced the understanding of mental illness from a medical perspective. He was one of the first individuals to propose that mental illnesses were rooted in biological origins (First, Frances, & Pincus, 2004).

Hippocrates came to this conclusion through detailed observations of individuals with pathological traits such as melancholia, hysteria, seizures, and mania. It is speculated that Hippocrates used the term “mania” in reference to what are now identified as the positive symptoms of schizophrenia (Angst & Marneros, 2001; Weinberger, 2011).

Many years later, during the 18th century psychotic symptoms were more clearly delineated and the term schizophrenia was coined. Two clinicians, Emil Kraepelin (1856-1926) and Eugene Bleuler (1857-1939) are widely credited with these advancements.

In 1919, Kraepelin published *Dementia Praecox and Paraphrenia*; this was a comprehensive description of the various and diverse behavioral abnormalities exhibited by individuals with psychosis. It is important to note that Kraepelin studied patients in a clinical setting, who were considered very ill (Kraepelin, 1971).

Based on his clinical observations, *Dementia Praecox and Paraphrenia* provided a conceptual framework for what later would be identified as the condition of schizophrenia. The term *dementia praecox* was used to indicate that this psychological illness manifests in adolescence (*praecox* is the Latin term for “early”) (Kraepelin, 1971; First et al., 2004; Weinberger, 2010).

Interestingly, Kraepelin observed that there were two disparate groups of individuals with psychosis. He differentiated the two groups by long-term clinical outcome. The first group was composed of individuals who experienced episodes of psychosis, but eventually went into remission. Kraepelin diagnosed the individuals in this group with manic-depression, today known as bipolar disorder.

The second group was composed of individuals with chronic psychosis, who unfortunately, experienced severe and permanent cognitive deterioration. The individuals in this group were diagnosed with dementia praecox (Kraepelin, 1971; Weinberger, 2010).

Although Kraepelin argued that both delusions and hallucinations were symptoms of schizophrenia, he believed that negative symptoms and chronic cognitive deterioration were key identifying features of the disease (Kraepelin, 1971, Jablensky, 2010; Weinberger, 2010). According to Kraepelin, negative symptoms included alogia, avolition, anhedonia, blunted affect and deficits in selective attention (Weinberger, 2010).

Like Kraepelin, Bleuler theorized that schizophrenia had key fundamental and secondary symptoms. He also believed that negative symptoms were key features of the disease (First et al., 2004). Bleuler described negative symptoms as deficits in associations, affect deregulation, autism, and ambivalence (Weinberger, 2010). However, Bleuler theorized that delusions and hallucinations were not defining characteristics of schizophrenia because they also occurred in other illnesses, such as bipolar disorder (Rosenzweig, Breedlove, & Watson, 2005).

In contrast to Kraepelin, Bleuler conceptualized that schizophrenia was not always associated with severe and permanent cognitive deterioration. This may be because Bleuler identified a subset of individuals with schizophrenia who were not as ill as the individuals that Kraepelin studied. He proposed that the condition of schizophrenia might have diverse biological origins, which in turn determine the severity of the affected individual's psychological wellbeing (Bleuler, 1950; Ashok, Baugh, & Yeragani, 2012).

Eventually, Bleuler coined the term schizophrenia, which replaced the term dementia praecox from clinical use. The term schizophrenia was chosen to indicate that affected individuals experienced a fragmentation of thoughts or a “splitting of the mind” (Jablensky, 2010). The German word “schizien” means split and “phren” is the Greek word for mind (Bleuler, 1950; Weinberger, 2010; Ashok et al., 2012).

Today, the condition of schizophrenia is identified by negative, positive and disorganized symptoms. These symptoms cause the individual to experience profound disruptions in processing emotions, cognitive tasks, and social interactions. It is estimated that worldwide, between one and three percent of the population suffers from schizophrenia. Schizophrenia is categorized under the Schizophrenia Spectrum and Other Psychotic Disorders section in the DSM-5 (DSM-5, 2013).

The diagnostic criteria for an individual to be identified as having schizophrenia necessitates that the individual experiences two or more of the following symptoms: delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behavior, or negative symptoms. The individual must be symptomatic for a significant portion of time over the course of one month (DSM-5, 2013).

The World Health Organization reports that schizophrenia is one of the top ten illnesses that contribute to the global burden of disease. The global burden of disease is a measure of mortality and disability caused by a given illness (Murray & Lopez, 1996; Weinberger, 2010; Tsuang, 2011).

Unfortunately, the World Health Organization estimates that half of individuals affected with schizophrenia do not receive appropriate care. This is of concern for many reasons, one of which is that individuals with schizophrenia are vulnerable to self-harm;

the suicide rate for individuals with schizophrenia is five times higher than that of non-affected individuals (Hor & Taylor, 2010).

In addition to individual and familial suffering, there are socio-economic burdens associated with schizophrenia, which are measured by direct and indirect costs. Direct costs are an estimate of the amount of money spent on one's medical treatment; this includes the cost of doctor's visits, hospital stays and the cost of medication, as well as any criminal justice expenses an affected individual may have accrued. Indirect costs are the estimated dollar amount of lost productivity and contribution to society by the affected individual. In 2002, in the United States, direct and indirect costs of schizophrenia management were estimated at 62.7 billion dollars (McEvoy, 2007).

The scientific community currently believes that an individual is likely to develop schizophrenia as a result of a complicated interplay between environmental and genetic factors (Bassett, Chow, O'Neill, & Brzustowicz, 2001). It is theorized that problems in environmental or genetic domains may negatively influence early neurological development, which later can lead to structural abnormalities in the adult brain, including the extracellular matrix. These neuroanatomical problems are postulated to result in the development of schizophrenia in adolescence or early adulthood (Weinberger, 2010; Berretta, 2012; Brown & Patterson, 2012).

Aloysius Alzheimer (1864-1915) and Franz Nissl (1860-1919) carried out the earliest documented biological investigations of schizophrenia in postmortem brain samples and were the first to report that there were neurobiological changes in the brains donated by individuals with schizophrenia (McKenna, 1997).

Alzheimer studied brain samples from individuals who were believed to have schizophrenia and also displayed catonic features. After these individuals passed away, Alzheimer removed their brains and cut the brain into thin slices. This method allowed Alzheimer to study and compare structural differences between brain samples donated by individuals with schizophrenia to the non-affected individuals.

Specifically, Alzheimer discovered that there were differences in the cerebral cortex in samples donated by individuals with schizophrenia. The cerebral cortex is usually two to four millimeters thick and is the outermost part of the brain. The cerebral cortex is involved in many cognitive processes including, thinking, attention, and consciousness (Bear, 2001, Kalia, Madhu, & Costa, 2015).

Most notably, Alzheimer found that in the brain samples recovered from individuals diagnosed with schizophrenia, many of the cells that make up the cerebral cortex were smaller, had wrinkled membranes, and their nuclei were swollen.

Like Alzheimer, Nissl also carried out postmortem studies on brain donations from individuals with schizophrenia; however, these individuals did not suffer from paralysis and Nissl did not see changes in cells from the cerebral cortex, like Alzheimer had previously reported. The presence of paralysis was the major difference between the groups and it was concluded that heterogeneous symptoms were caused by diverse neurobiological abnormalities (Kraepelin, 1971; Akbarian et al., 1996; Galtrey & Fawcett, 2007; Benes & Berretta, 2001).

Although, the first documented accounts of neuronal abnormalities in schizophrenia were delineated in the 19th century, it wasn't until the 1950's that pharmacological interventions for individuals with schizophrenia were developed. The

first medication engineered to treat schizophrenia was developed in 1951. The drug was called Chlorpromazine and it was sold as Thorazine (Kandel, 2013).

Collectively, the drugs that were produced soon after were termed first-generation antipsychotics or typical antipsychotics (Pies, 2005). They were effective in alleviating the positive symptoms experienced by individuals with schizophrenia, but had little effect on negative symptoms. Unfortunately, individuals taking first-generation antipsychotics were prone to experience various adverse side effects (Shiloh, 2006; Ellenbroek, 2012).

A study of the molecular mechanisms of first-generation antipsychotics in animal models revealed that these drugs lowered levels of the neurotransmitter dopamine. This observation gave way to the dopamine hypothesis of schizophrenia, which stated that elevated levels of dopamine were responsible for the positive symptoms an individual with schizophrenia may typically experience (Pies, 2005; Weinberger, 2010).

Developed in the 1990s, Clozapine was one the first of a new class of antipsychotic medications called second-generation antipsychotics or atypical antipsychotics. The second-generation antipsychotics are a diverse group of compounds that are considered atypical because they do not cause extrapyramidal side effects with the severity of the first-generation antipsychotics (Tuunainen, Wahlbeck, Simon, 2002). However, second-generation antipsychotics can produce side effects that include life threatening metabolic changes (Peluso, Lewis, Barnes, & Jones, 2012; Gautam & Meena, 2011).

Although, first-generation and second-generation antipsychotics are used worldwide to treat the positive symptoms associated with schizophrenia, these drugs are

less effective in the treatment of socio-cognitive deficits and the negative symptoms that individuals with schizophrenia experience (Lally & MacCabe, 2015).

Recent focus in neuroscience research has honed in on a region of the brain, known as the amygdala, which when structurally impaired may contribute to the manifestation of certain symptoms individuals with schizophrenia experience (Berretta, 2012; Pantazopoulos, et. al, 2015).

The amygdala is responsible for certain cognitive functions that are impaired in individuals with schizophrenia, specifically, the amygdala enables an individual to perform socio-cognitive processes and regulate emotional behavioral responses. Often, individuals with schizophrenia show deficits in these domains (Pinkmam, 2015).

Much of the amygdala's role in schizophrenia comes from functional imaging studies that show altered neuronal activation in several anatomical regions, including the amygdala. It is proposed that increased amygdala activity in individuals with schizophrenia may be a contributing factor to the individual's feelings of heightened anxiety and paranoia, which ultimately can lead to one's withdraw from social behavior (Phelps and Ledoux, 2005; Pinkmam, 2015).

More specifically, imaging studies have shown that individuals with schizophrenia, compared to non-affected individuals, have increased amygdala activity in response to viewing negative affective stimuli and decreased amygdala activation during observation of positive stimuli. These imaging studies supported previous subjective reports by individuals with schizophrenia who indicated that they experience more negative emotions compared to unaffected individuals (Beck & Holmes, 2011; Pankow, et al., 2013).

The amygdala works with other brain regions to regulate an individual's attention, evaluation, and response to sensory information. Some of the amygdala's major functions are to enable an individual to evaluate their environment, identify potential threats, and initiate behavioral responses aimed to protect the individual from harm. This indicates that the amygdala not only increases the individual's probability of survival but also that of the species (de Gelder et al., 2014)

However, hyperactive amygdala activity, as proposed is the case in individuals with schizophrenia, can be detrimental. One example of this is that affected individuals are prone to sense danger in response to non-threatening stimuli. This can lead to non-adaptive behavioral responses, such as paranoia, social withdraw or aggression (Bear, 2001; Pankow, 2013).

Another, important socio-cognitive tasks that the amygdala facilitates is the ability to identify fear (expressed by facial expressions) in other individuals. The amygdala's role in fear assessment is illustrated in the following two experiments (Adolphs, 2002).

In the first experimental paradigm, researchers used an electrical current to stimulate the amygdalae of patients during brain surgery. When the amygdala was stimulated patients often reported feelings of fear and a sense of imminent danger. In the second experimental paradigm, researchers studied individuals whose amygdalae were damaged by stroke. These individuals were able to identify most facial expressions, but not fearful ones (Aggleton, 1993; Adolphs, 2002; Gur et al., 2002).

This is an especially important area of focus because socio-cognitive factors such as the ability to accurately ascertain emotions in facial expressions are impaired in

individuals with schizophrenia and hinder affected individuals in engaging in goal oriented behaviors (Butcher, Mineka, & Hooley, 2007; Clark, Gosselin, & Goghari, 2013).

Because the amygdala enables individuals to carry out socio-cognitive functions such as attention, decision-making, and execution of behaviors and individuals with schizophrenia show impairments in these domains (Sergeyev, Chochol, & Armony, 2008) the focus of this thesis will be centered on neurons found in the amygdala.

More specifically, this thesis will focus on GABAergic neurotransmission in the amygdala, which has been associated with cognitive impairments in individuals with schizophrenia (Wassef, Baker, & Kochan, 2003; Bernstein, 2007; Berretta, 2009; Lally & MacCabe, 2015). GABAergic neurotransmission is inhibitory and a lack of inhibition of excitatory neurotransmission may explain why the amygdala may be hyperactive in response to neutral stimuli (Pankow, 2013).

Hampered GABAergic neurotransmission was first documented in individuals with schizophrenia who had disturbances in gamma-frequency neuronal synchrony (Perry, 1979; Uhlhaas & Singer, 2010; Nakazawa, 2012). This type of neural oscillation occurs during cognitive processes, which include: attention, memory formation, and consciousness. A specific subtype of GABAergic interneurons facilitates gamma-frequency neuronal synchrony; these neurons express the protein parvalbumin (PV+ GABAergic interneurons) (Williams & Boksa, 2010).

In order for the brain to generate gamma-frequency neuronal synchrony, excitatory neurons are inhibited by PV+ GABAergic interneurons (Bartos, Vida, & Jonas, 2007). PV+ GABAergic interneurons are impaired in individuals with

schizophrenia and are not able to inhibit excitatory neurons (Benes & Berretta, 2001) as a result, gamma-frequency neuronal synchrony does not occur.

The inability of PV+ GABAergic interneurons to effectively inhibit excitatory neurons may be caused by problems with the structures that surround PV+ GABAergic interneurons. This area is known as the extracellular matrix, which has numerous functions which include: providing structural support for neurons and glia, guiding cells during neurogenesis, helping to regulate cell-to-cell communication, and regulating neuronal plasticity (Pantazopoulos et al., 2010; McRae & Porter, 2012).

This is significant because the condition of schizophrenia may be predicated on disrupted neuronal cell migration during development and neural connectivity issues in adulthood (Pantazopoulos et al., 2010; Berretta, 2011; Berretta, Pantazopoulos, Markota, Brown, & Batzianouli, 2015).

A recent postmortem study showed that there were fewer perineuronal nets (condensed extracellular matrix structures) surrounding PV+ GABAergic interneurons in the amygdala of subjects with schizophrenia compared to control subjects (Pantazopoulos et al., 2010; Sah & Lodge, 2013).

It is important for neurons to be surrounded by perineuronal nets because a reduction in perineuronal nets may impair the inhibitory functions of PV+ GABAergic interneurons. This may be because perineuronal nets protect neurons from oxidative stress and help to maintain sodium and potassium levels inside nerve cells. Therefore, if perineuronal nets are missing or not formed correctly, the neuron is left unprotected from damage caused by oxidative stress. In addition sodium and potassium imbalances may

occur and the neuron's firing ability may be compromised (Yamaguchi, 2000; Cabungcal et al., 2013).

There is evidence that the protein Otx2 is needed for the formation of nets during early postnatal brain development; Otx2 expression coincides with the formation of perineuronal nets. Perineuronal net formation around neurons results in the closing of neuro-critical periods of development. A reduction in the expression of Otx2 may result in fewer or structurally impaired perineuronal nets (Sugiyama, Prochiantz, & Hensch, 2009).

Otx2 belongs to a group of proteins called transcription factors; these proteins are able to guide many biological processes, including neuronal development, by activation of various genes needed in the given processes. Development is a highly controlled event and schizophrenia may be a consequence of impaired neurological development (Dragan et al., 2006; Housset et al., 2013).

Otx2 is a concrete example of a protein that facilitates developmental processes, as Otx2 is first active during embryogenesis, but Otx2 reemerges in postnatal periods of development. During embryogenesis, Otx2 is needed for the formation of specific brain regions and later, in postnatal stages, Otx2 is needed to drive the maturation of PV+ GABAergic cells. Once PV+ GABAergic cells mature, perineuronal nets are able to form around and stabilize neurons (Soleman, Filippov, Dityatev & Fawcett, 2013). In the absence of Otx2, PV+ GABAergic maturation and perineuronal net formation are hindered (Beurdeley, 2012).

Much of Otx2's role in embryogenesis has been characterized in mice; upon deletion of Otx2 mice fail to develop forebrain and midbrain regions. Thus, Otx2 is vital

for the survival of the mouse (Li & Joyner, 2001). Otx2 is needed throughout the life of the mouse. For instance, in the mouse visual cortex at post-natal day19, before critical period onset, little Otx2 protein is evident. However, during the peak of the visual critical period, there is an increase in Otx2 along with the stabilization of PV+ GABAergic interneurons (Sugiyama et al., 2009).

Similarly, in humans, Otx2 is found in several brain regions during development. The presence of Otx2 in these areas indicates that Otx2 also has a functional role in human neurodevelopment. Furthermore, the absence of Otx2 results in Otocephaly, a lethal condition in which one's jawbone does not develop (Larsen, Lutterodt, Mollgard, & Moller, 2010).

In later postnatal stages of development Otx2 is not produced by PV+ GABAergic interneurons despite evidence that Otx2 is needed to maintain mature PV+ GABAergic interneurons; therefore, the source of the protein Otx2 that aids in the formation of perineuronal nets around PV+ GABAergic interneurons originates elsewhere. Otx2 messenger RNA that gives rise to the protein Otx2 is not found in PV+ GABAergic interneurons but it is found in the choroid plexus. This indicates that Otx2 used to maintain mature PV+ GABAergic interneurons might be produced in the choroid plexus (Spatazza et al., 2013).

Taken together with evidence of GABAergic dysfunction in schizophrenia, it makes sense to hone our examination to PV+ GABAergic interneurons located in the amygdala. We propose that Otx2 protein is made in the choroid plexus, and then packaged in vesicles that are transported in the cerebral spinal fluid, through the lateral ventricle to the amygdala.

Once Otx2 reaches the amygdala it is able to facilitate the formation of perineuronal nets, which will form around mature PV+ GABAergic interneurons. This process facilitates the proper functioning of PV+ GABAergic interneurons and we propose that this mechanism is disrupted in individuals with schizophrenia.

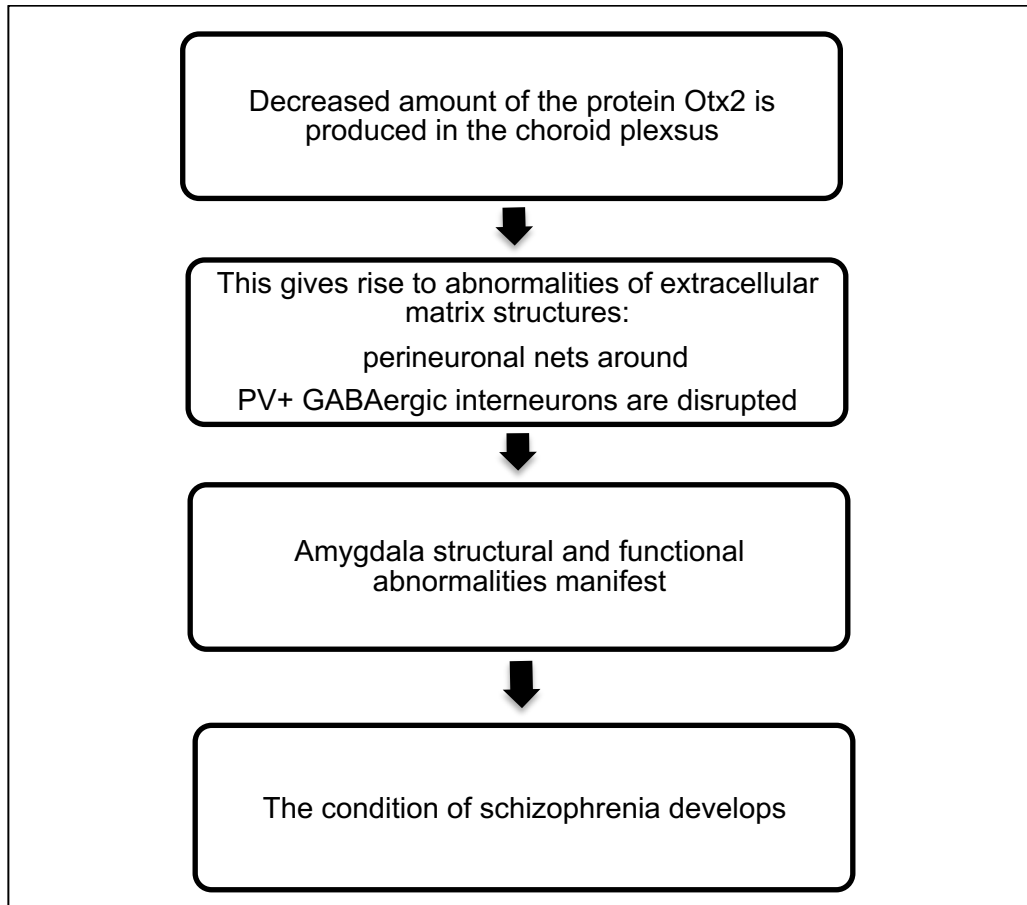


Figure 1. Proposed molecular underpinnings of the condition of schizophrenia

Chapter II

Research Method

All post mortem tissue used in this project was received from the Harvard Brain Tissue Resource Center. Prior to donation, consenting donors fill out the appropriate documentation and register with the Harvard Brain Tissue Resource Center, a National Institute of Health affiliated brain repository. Brain donations may also be obtained from individuals who are registered organ donors. When the donor passes away, the brain repository is notified and the process of donation collection begins.

Brain collection is a tightly regulated process. First, a qualified professional such as a mortician or a trained physician removes the donated brain. In attempt to delay degradation of the donated brain, the collection is carried out as quickly as possible. Next, the donated brain is packaged, placed on ice, and shipped; a specialized courier service is used to deliver the brain to the Harvard Brain Tissue Resource Center located at Mclean Hospital in Belmont, Massachusetts.

Once the brain arrives at Mclean Hospital a trained dissectionist will identify and cut out designated brain regions. These specific regions have been selected because previous scientific investigation has indicated that abnormalities in these areas are correlated to various disease states. Disease areas of interest include Parkinson's disease, schizophrenia, bipolar disorder, and Huntington's disease.

Upon removal the brain is first cut in half, separating the left and right hemispheres. One hemisphere is immediately placed in formalin to be preserved for later

scientific study. This will occur after a pathologist confirms the donor's medical diagnosis. The other hemisphere will be dissected, frozen and stored at negative eighty degrees Celsius until it is needed for research.

Amygdala preservation by freezing

For the experiments carried out in this proposal, I used frozen amygdala tissue samples and cerebral spinal fluid donations. The amygdala and cerebral spinal fluid samples were prepared using different methods. Amygdala tissue samples were frozen and cut prior to experimentation, whereas cerebral spinal fluid samples required less processing prior to experimentation. A small volume of cerebral spinal fluid was taken dried directly onto a microscope slide without prior freezing.

In general, freezing biological specimens is a harsh process that can damage the specimen. To freeze the amygdala without damaging it, the amygdala was submerged in a plastic container that contained O.C.T. O.C.T stands for Optimal Cutting Temperature compound. It is a viscous liquid that upon freezing will harden around the tissue and will protect it from damage.

Next, the container with the amygdala floating in O.C.T was submerged into liquid nitrogen. Submersion of the amygdala into liquid nitrogen allowed for rapid freezing, which is especially important because rapid freezing in O.C.T reduces damage to the tissue.

Next, the frozen amygdala sample, ensheathed by O.C.T., was placed into a cryostat for slicing. The cryostat is a cold chamber that houses a very sharp blade that is designed to cut biological samples into extremely thin sections. The sample was cut into

slices of approximately twenty micrometers in width. This is necessary because when the sample is observed with a microscope, light must be able to penetrate through the sample to enable detailed observation of micro-anatomical structures.

After the amygdala was cut into thin sections, the sections were placed into small, plastic containers and standard immunohistochemical (IHC) procedures were carried out. Upon completion of the IHC procedure, vesicles that contained Otx2 were visible and were counted using computer an assisted light microscope and Stereo Investigator software.

IHC to visualize Otx2 in vesicles for quantification

Before completion of the IHC reaction, prepared amygdala slices were very light in appearance; when observed with a light microscope they were almost entirely translucent and the vesicles that contain Otx2 could not be seen. In order to visualize the vesicles that contained Otx2, a specific antibody was used to detect Otx2 in both the amygdala and cerebral spinal fluid samples. Later, a chemical reagent that turned the Otx2-antibody complex brown was applied. This rendered the vesicles that contained Otx2 visible to count.

In general, IHC is performed when undetectable, microscopic structures in biological samples need to be identified. It is a multi-step technique that uses a series of antibodies and an enzymatic reaction to visualize the protein of interest by turning it dark brown or black.

In these IHC experiments, the first step was to utilize an antibody that recognized and attached to Otx2. In essence, this antibody was used to locate a vesicle filled with

Otx2 within the sample. The first antibody, called the primary antibody, was added to the buffer in which the amygdala was floating in and directly onto the slide that contained dried cerebral spinal fluid. The same primary antibody was used in both amygdala and cerebral spinal fluid IHC procedures; it was purchased from Abcam (Catalog number: ab76748). After the primary antibody attached to Otx2, a second antibody was used to attach to the primary antibody. Like the primary antibody, the secondary antibody will only bind to specific designated structures; in this case it will only bind to the primary antibody that attached to Otx2.

The secondary antibody was purchased from Vector Laboratories (Catalog number: PI-1000). After the secondary antibody attached to the primary antibody, a chemical called 3, 3'-diaminobenzidine, commonly abbreviated DAB, and hydrogen peroxide were added to the amygdala and cerebral spinal fluid samples. Horseradish peroxidase, attached to the secondary antibody, reacted with hydrogen peroxide to alter the chemical DAB and turned it brown. Brown DAB could now be used to identify where the secondary antibody had attached to the primary antibody. This was the site of Otx2 in the sample.

After the IHC assay was performed on amygdala samples, the sections were manually guided onto a glass microscope slide, using a paintbrush. Two amygdala slices were placed on each glass microscope slide. Amygdalae vary in size, but approximately forty, twenty-micrometer slices were generated from one amygdala.

Quantification of vesicles that contain Otx2 in the amygdala

Vesicles that contained Otx2 were manually counted using a computer assisted light microscope and Stereo Investigator software. The software is advantageous because it allows the user to mark objects and keep track of objects in the tissue sample. In addition, the software estimates the size of any given area traced by the user. This allows the user to determine the number of marked objects in a given area and compare the number of marked objects between samples per given area size.

In the amygdala, the following regions were examined for the presence of vesicles that contained Otx2: the lateral, basal, accessory basal, medial, central and cortical nuclei. The total area of the amygdala and area of each subdivision of the amygdala was identified, measured, and recorded. The number of vesicles that contained Otx2 in a given area was counted.

The following sampling system was developed to accelerate the counting process: first, all the vesicles that contained Otx2 were counted in one entire amygdala sample and the numbers of vesicles in each region of the amygdala were documented. Next, by trial and error, the Stereo Investigator software was programmed with the correct parameters that generated a sampling system that matched the original count. This included manipulation of the size of the sampled area and number of sample sites.

Step-wise regression analysis was performed to determine if there were significant differences in the number of vesicles that contained Otx2 between schizophrenic and control groups. This statistical method took in to account other variables besides disease diagnosis that may influence the results. These included: the individual's gender, age, and use of antipsychotics.

Preparation of cerebral spinal fluid for IHC

To detect Otx2 in the cerebral spinal fluid samples, a small amount of cerebral spinal fluid was removed from a post mortem brain donation. After the dissectionist cut the brain in half, separating the two hemispheres, the lateral ventricle was accessible and cerebral spinal fluid was collected with a small device designed to vacuum the fluid out of the ventricle and into a small tube. Approximately two milliliters of cerebral spinal fluid was available for collection. Forty micrometers of cerebral spinal fluid was aliquoted onto a slide and allowed to dry, then stained for identification of Otx2 using the same IHC protocol used for vesicle visualization in amygdala samples.

Cerebral spinal fluid and ELISA

Enzyme-linked immunosorbent assay (ELISA) is a way to measure the amount of protein, such as Otx2, within a sample. The principles described above for the IHC are the same in the ELISA assay. The ELISA kit was purchased from Cusabio (Catalog number: CSB-EL017299HU). In brief, a small amount of cerebral spinal fluid was placed into a well where an antibody that binds to Otx2 is attached to the bottom; the sample was incubated for one hour. After this incubation step, the well was washed with a buffer and the components of the sample not attached to the antibody were washed away, immobilizing Otx2 to the bottom of the well.

Like in the IHC assay, a secondary antibody was added after the primary antibody incubation. This antibody also had horseradish peroxidase attached to it; however, a different chemical was used to induce a color change that identified Otx2. 3, 3', 5, 5'-

Tetramethylbenzidine (TMB) was used and turned Otx2 a blue color and finally, an acidic reagent was added that stopped the chemical reaction and turned the sample yellow. A spectrophotometer was used to estimate the amount of Otx2 in each sample based on the samples color saturation. The lighter yellow samples contained lower levels of Otx2.

A t-test was performed to determine if there were significant decreases in the amount of Otx2 in the samples donated by individuals with schizophrenia.

Chapter III

Results

Experiments carried out for this thesis were based on observation and quantification of vesicles that contained the protein Otx2 in post mortem amygdala and cerebral spinal fluid samples.

Vesicles in the amygdala contained Otx2

In order to visualize Otx2, IHC experiments utilizing DAB were performed. This turned vesicles containing Otx2 a dark color. This experimental procedure revealed the presence of numerous vesicles that contained Otx2 in amygdala samples donated by healthy control subjects (Figure 2) and by individuals with schizophrenia (Figure 3).



Figure 2. Vesicles contained Otx2 in samples donated by healthy controls. This image was magnified ten times.

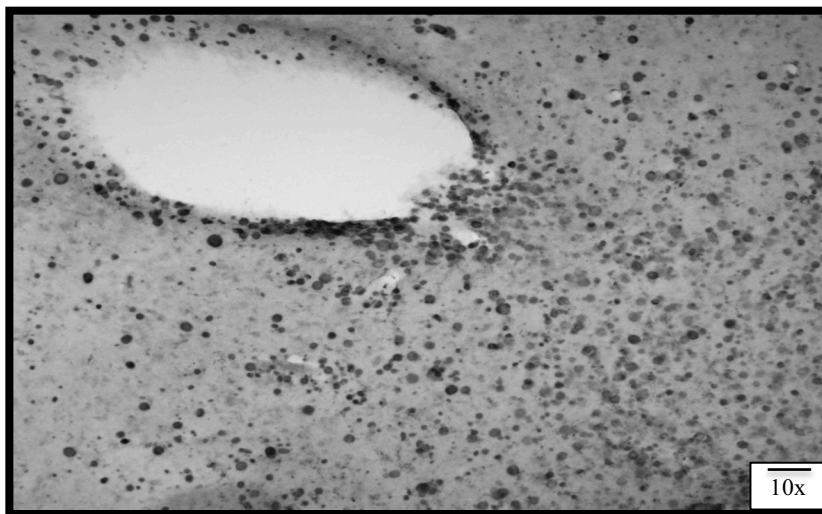


Figure 3. Vesicles contained Otx2 in samples donated by individuals with schizophrenia. This image was magnified ten times.

The next step in this project was to count the number of vesicles that contained Otx2. Once all of the samples were counted, the number of vesicles was compared between samples donated by individuals with schizophrenia and the control group.

During this process, several interesting observations were made about the quality, quantity and distribution of vesicles that contained Otx2 in samples donated by both individuals with and without schizophrenia.

For example, vesicles filled with Otx2 were concentrated in a specific region of the amygdala; the highest concentration of vesicles that contained Otx2 in the amygdala was found adjacent to the lateral ventricle. Moreover, the density of vesicles that contained Otx2 decreased moving away from the ventricle and into the tissue. This observation was made in amygdala samples donated by both healthy controls (Figure 4) and by individuals with schizophrenia (Figure 5).

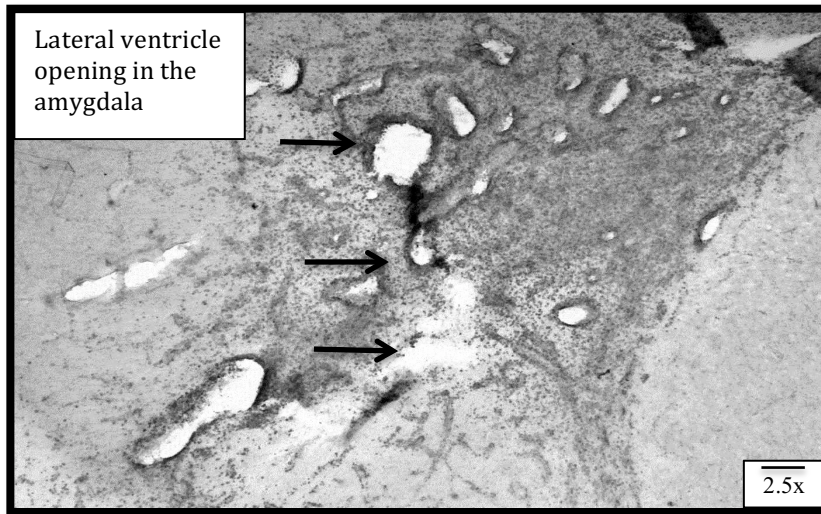


Figure 4. Vesicles near the lateral ventricle in healthy controls. This image was magnified two and a half times.

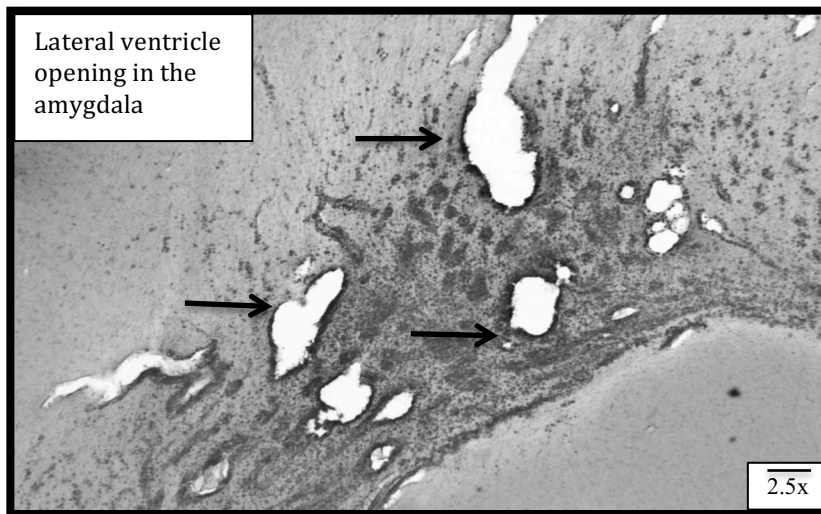


Figure 5. Vesicles near the lateral ventricle in individuals with schizophrenia. This image was magnified two and a half times.

Furermore, vesicles containing Otx2 were found in the lateral ventricle in amygdala samples donated by the control group (Figure 6) and in samples from individuals with schizophrenia (Figure 7).

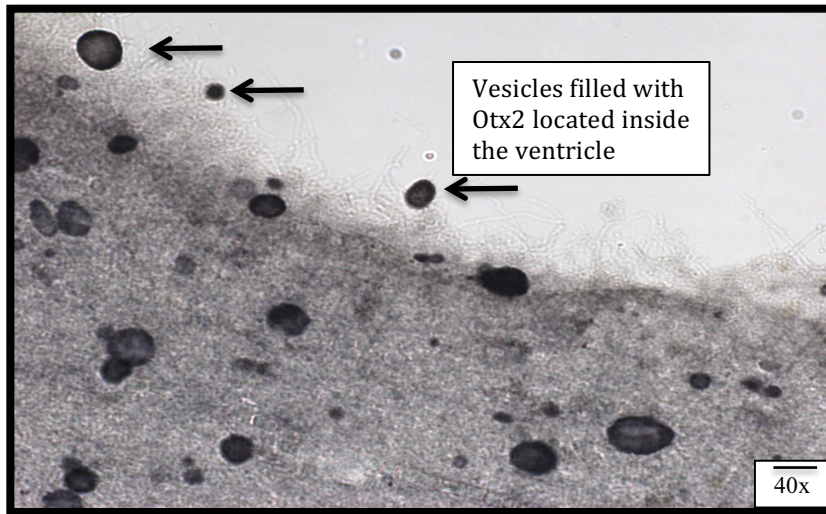


Figure 6. Vesicles inside the ventricle in donated healthy control samples. This image was magnified forty times.

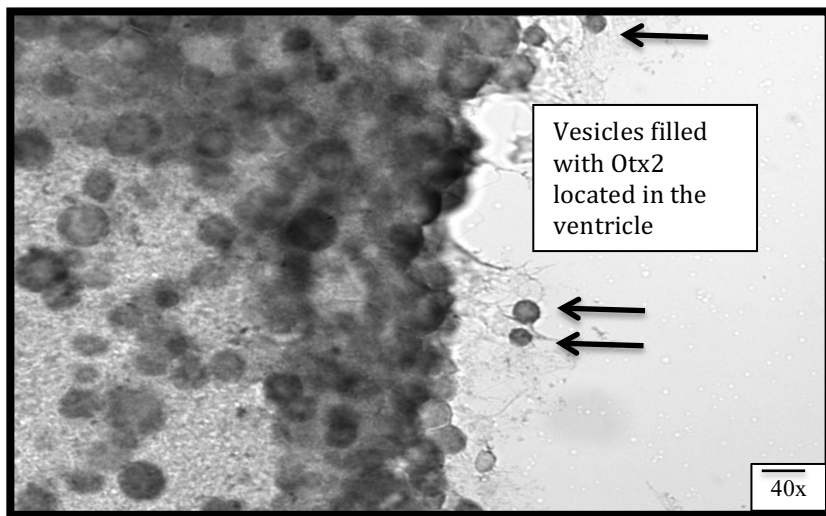


Figure 7. Vesicles inside the ventricle in donated schizophrenia samples. This image was magnified forty times.

In addition, vesicles that contained Otx2 were observed to be heterogeneous with respect to color and size. In samples donated by healthy controls and by individuals with schizophrenia, vesicles ranged from light gray to black (Figure 8 and Figure 9) and also showed differences in size (Figure 10 and Figure 11).

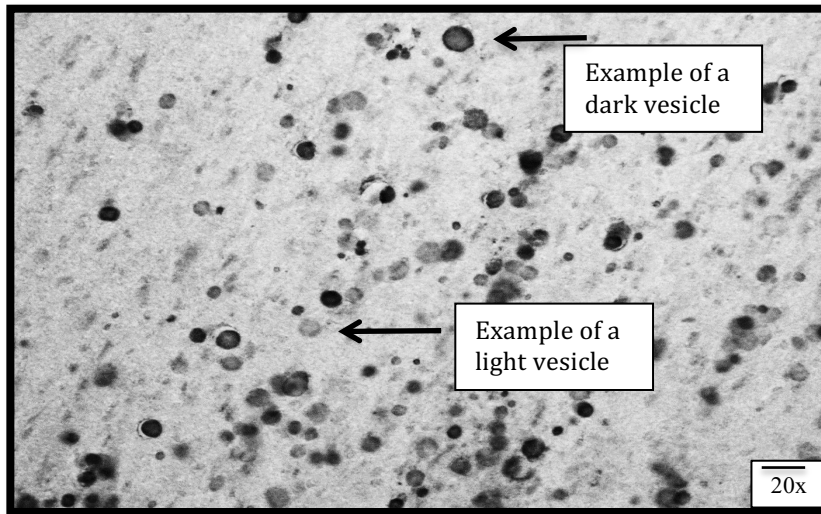


Figure 8. Light and dark vesicles in samples donated by healthy controls. This image was magnified twenty times.

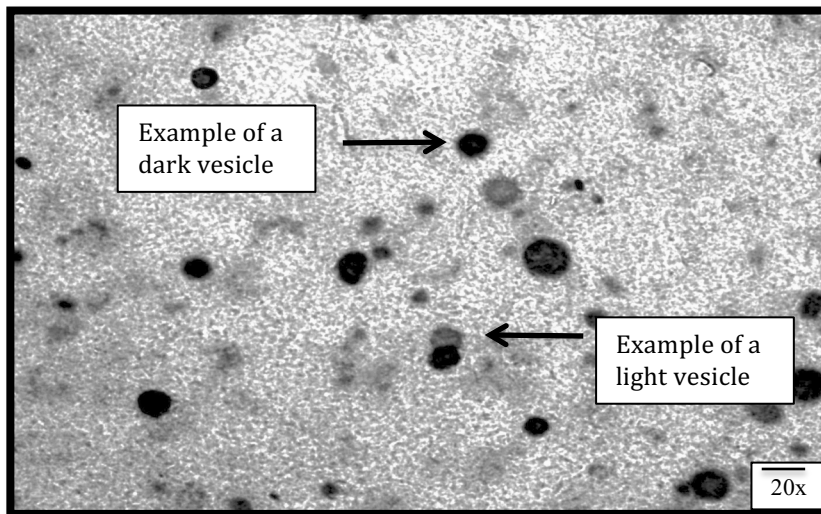


Figure 9. Light and dark vesicles in samples donated by individuals with schizophrenia. This image was magnified twenty times.

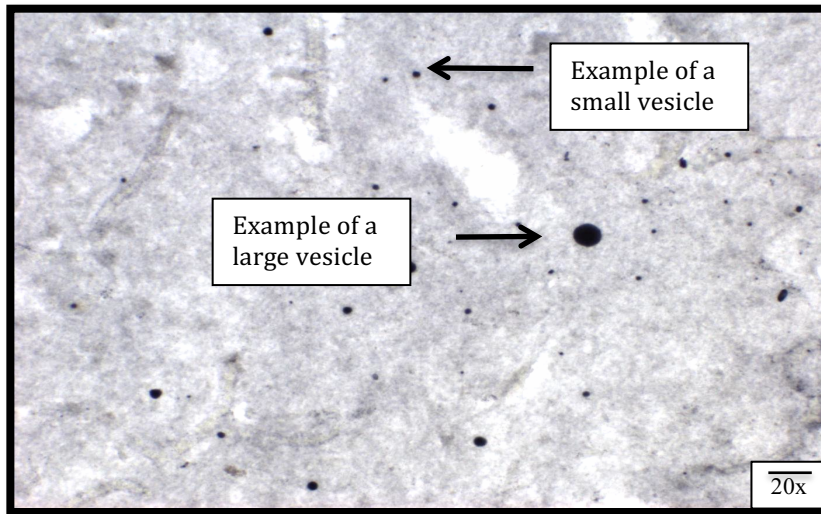


Figure 10. Vesicles varied in size in samples donated by healthy controls. This image was magnified twenty times.

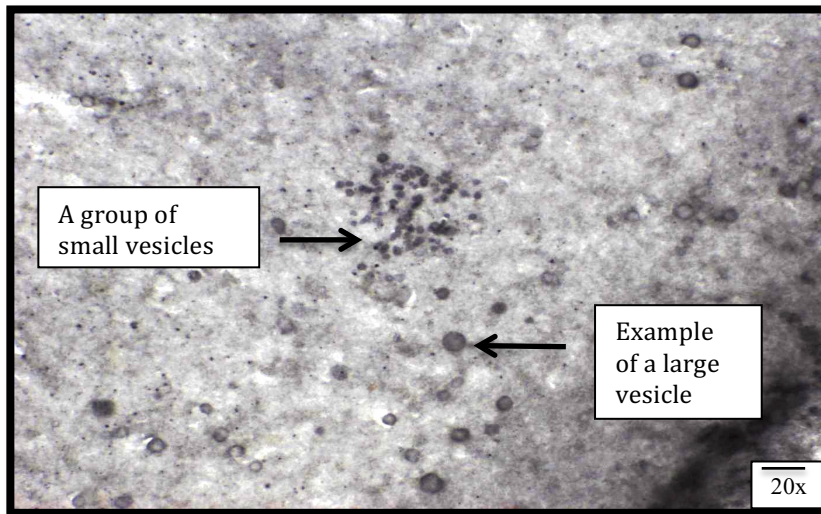


Figure 11. Vesicles varied in size in samples donated by individuals with schizophrenia. This image was magnified twenty times.

Finally, a subset of vesicles in the amygdala and lateral ventricle appeared to be hollow; it appeared that an outline of a vesicle was present, however, it was a translucent structure. This was observed in samples donated by healthy control subjects (Figure 12) and in samples donated by individuals with schizophrenia (Figure 13).

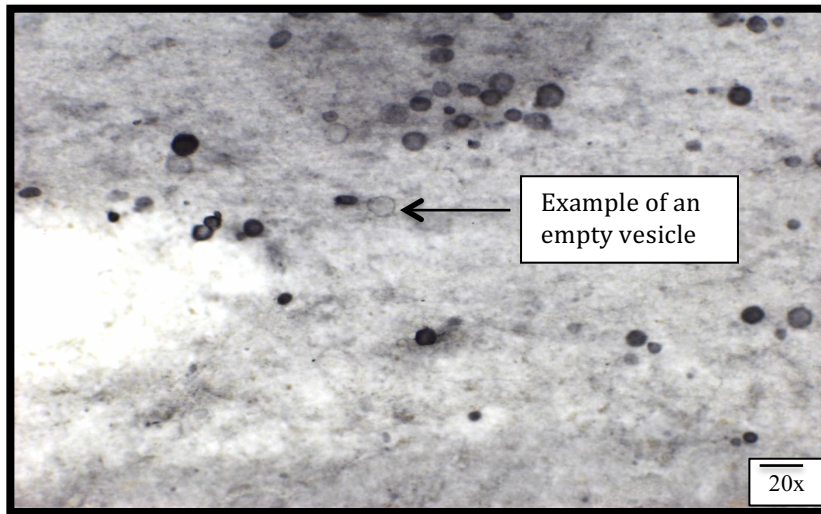


Figure 12. Hollow vesicles in samples donated by healthy controls. This image was magnified twenty times.

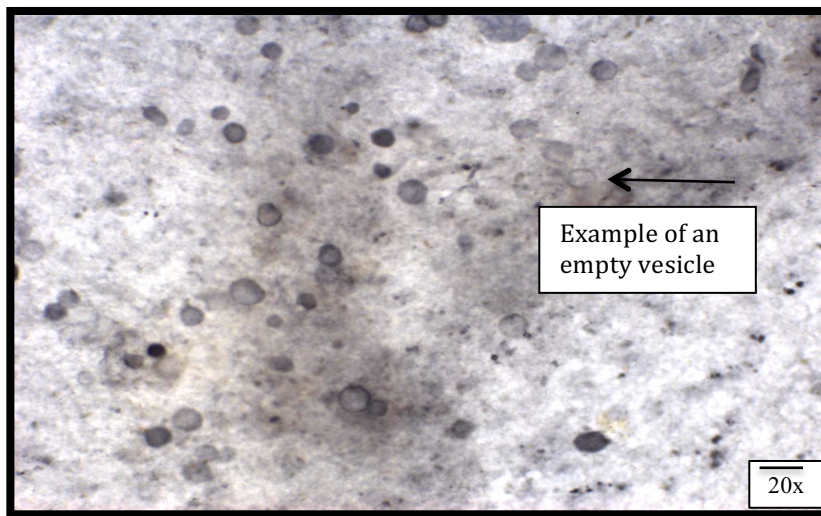


Figure 13. Hollow vesicles in samples donated by individuals with schizophrenia. This image was magnified twenty times.

Statistical Analysis

Step-wise regression analysis determined that there was not a significant difference between the numbers of vesicles found in samples donated by individuals with schizophrenia compared to the healthy control groups (p -value <0.05).

Vesicles in cerebral spinal fluid samples contained Otx2

In addition to the amygdala, vesicles that contained Otx2 were detected in samples of cerebral spinal fluid (Figure 14). In the first set of experiments, in addition to vesicles, cells that contained Otx2 were also detected in the cerebral spinal fluid samples. These cells also contained Otx2. The presence of vesicles that contain Otx2 in the cerebral spinal fluid was a novel finding.

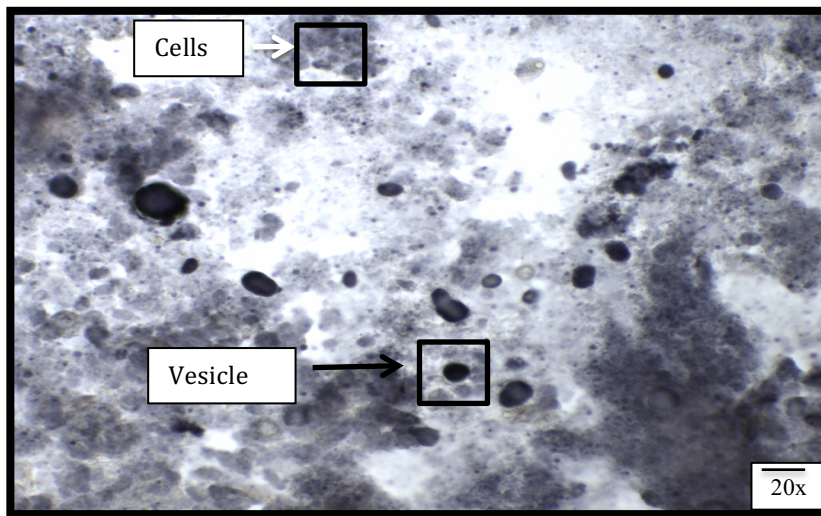


Figure 14. Vesicles and cells contained Otx2 in cerebral spinal fluid samples. This image was magnified twenty times.

Cells that contained Otx2 in cerebral spinal fluid samples were presumed to be a post mortem artifact. This thesis focused on vesicles that contained Otx2; therefore, vesicles were isolated from the cells using centrifugation.

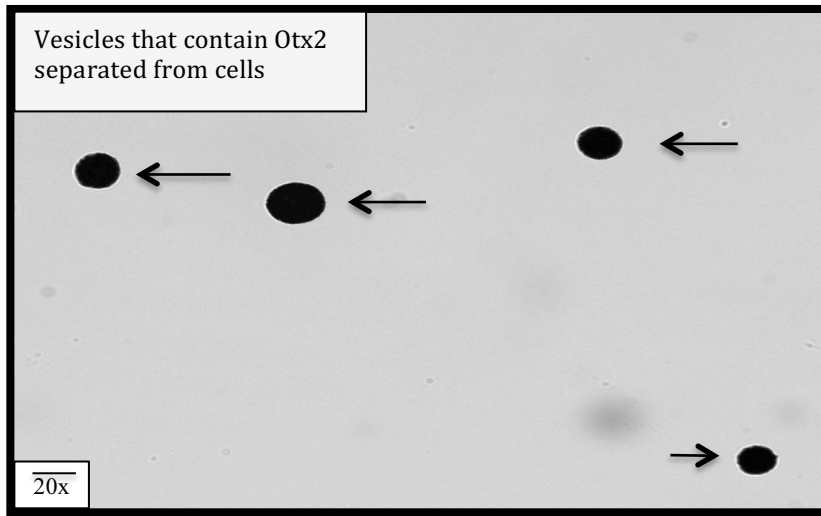


Figure 15. Vesicles in cerebral spinal fluid samples after cells were removed. This image was magnified twenty times.

Experiments performed on cerebral spinal fluid revealed approximately five vesicles that contained Otx2 were present per forty microliters of cerebral spinal fluid.

An ELISA assay performed on cerebral spinal fluid samples revealed that there was a significant decrease in the concentration of Otx2 in samples donated by individuals previously diagnosed with schizophrenia compared to samples donated by healthy control individuals ($p\text{-value} > 0.05$)(Table 1 & 2, & 16).

Two-sample T for Scizophrenia_Otx2 (ng/ml) vs Control_Otx2 (ng/ml)

Difference = mu (Scizophrenia_Otx2 (ng/ml)) - mu (Control_Otx2 (ng/ml))

Estimate for difference: -150.0

95% CI for difference: (-217.3, -82.6)

T-Test of difference = 0 (vs not =): T-Value = -4.62 P-Value = 0.000 DF = 22

Table 1. Summary of paired t-test results from comparison of vesicle number between samples donated by individuals with schizophrenia and healthy controls.

	Number of samples	Mean amount of Otx2 (ng/ml)	Standard Deviation	Minimum	Maximum
Control	14	307.7	86	183.4	456.3
Schizophrenia	11	157.7	76.1	72.9	270.6

Table 2. Descriptive statistics

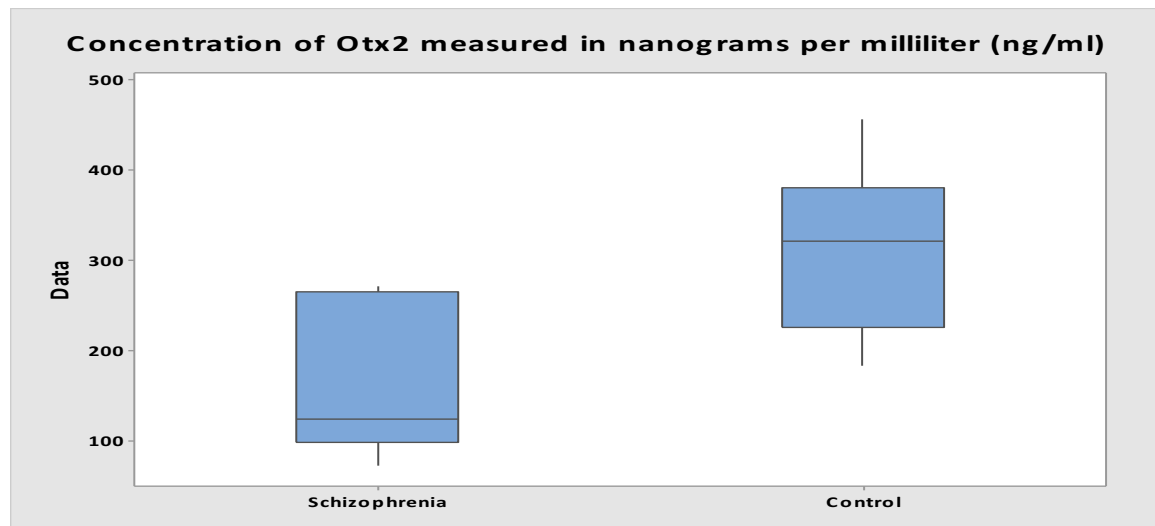


Figure 16. Decrease in Otx2 levels in individuals with schizophrenia

Chapter IV

Discussion

Schizophrenia is a chronic and debilitating neuropsychiatric condition that has likely affected individuals throughout history (Bark, Varadi, & Sorrentino, 2008; Weinberger, 2010). Currently, it is estimated that 1 to 3% of the population worldwide is affected with schizophrenia (DSM-5, 2013).

Accurate diagnosis of schizophrenia can be difficult to ascertain because, in large part, clinicians make diagnoses based on an individual's subjective description of their symptoms (Jablensky, 2010). At this time objective biological tests to identify schizophrenia are not available. In theory such tests would give a doctor the ability to diagnose an individual with schizophrenia based on objective biological criteria.

Ideally, biological testing, in corroboration with traditional diagnostic methods for identifying schizophrenia would result in faster and more accurate disease diagnosis (Hayes, Robinson, Muller, & Wang, 2009).

Improvements in disease diagnosis are important because individuals with schizophrenia, when diagnosed earlier, tend to respond better to treatment. McGlasshan argues that it would be ideal to identify individuals in the prodromal phase of schizophrenia and begin early treatment interventions. This strategy is predicted to result in the best clinical outcomes for affected individuals (McGlasshan, 2005; Schultz, 2007; Lake, 2012).

The first step in the development of a biological test to screen for schizophrenia is to elucidate the molecular underpinnings of the condition. With this in mind, the focus of this thesis was on a specific protein called Otx2 and its role in the pathophysiology of schizophrenia. The main hypothesis tested in experiments conducted for this thesis work was that individuals with schizophrenia would have lower levels of the protein Otx2 compared to healthy control subjects.

This was predicted because Otx2 is required for the development and maintenance of PV+ GABAergic interneurons and this class of neurons has functional deficits in individuals with schizophrenia (Bartos, Vida, & Jonas, 2007; Berretta, 2012; Cabungcal et al, 2013). It may be that a paucity of Otx2 is in part responsible for these functional deficits.

Furthermore, not only is Otx2 required for the initial development of healthy PV+ GABAergic interneurons, but it is also involved in the stabilization of mature PV+ GABAergic circuits (Beurdeley, 2012). Mature PV+ GABAergic interneurons are ensheathed by perineuronal nets and it has been shown that Otx2 is needed to maintain the structural integrity of these nets. Without stable nets, PV+ GABAergic interneurons are susceptible to damage (Benes & Berretta, 2001; Cabungcal, 2012).

Otx2's precise role in neuronal development and circuitry stabilization processes has not been fully elucidated. Interestingly, mature PV+ GABAergic interneurons do not synthesize Otx2 despite their need for it. It appears that Otx2 is transported from another site; evidence from mouse models indicates that cells located in the choroid plexus produce Otx2 and PV+ GABAergic interneurons in the amygdala utilize choroid plexus derived Otx2 (Beurdeley et al., 2012; Spatazza et al., 2013).

Furthermore, experiments in mouse models showed that when Otx2 production was disrupted in the choroid plexus, PV+ GABAergic interneurons in the amygdala malfunctioned (Beurdeley, 2012).

In experiments carried out for this thesis work, Otx2 was found in cells in cerebral spinal fluid samples. It is possible that these cells were derived from the choroid plexus because the choroid plexus has been shown to shed cells into the cerebral spinal fluid (de Reuck & Vanderdocht, 1986).

This finding is supported by mouse model evidence that Otx2 is produced in the choroid plexus. However, future experiments would need to be carried out to positively determine that these cells originate in the choroid plexus. Post-mortem cerebral spinal fluid may contain other cell types such as inflammatory cells (Morris, Harrison & Telford, 2012).

More definitive evidence that Otx2 is produced in the choroid plexus was recently seen in human postmortem samples in our laboratory. Otx2 mRNA, the precursor for protein Otx2, was found in the choroid plexus and importantly, Otx2 precursor mRNA was not found in the amygdala (Pantazopoulos et al., Manuscript in Preparation). This finding also supports the mouse model data, which indicates that neurons in the amygdala utilize Otx2 derived from the choroid plexus (Beurdeley et al., 2012).

It has not been established how Otx2 travels from the choroid plexus to the amygdala. Our finding of vesicles filled with Otx2 in the cerebral spinal fluid is a novel finding that may explain how Otx2 arrives in the amygdala from its site of synthesis in the choroid plexus (Beurdeley et al., 2012).

It is known that cells communicate with one another using various types of vesicles that contain signaling molecules. Currently, research has categorized several types of extracellular vesicles. These include: exosomes, micro-vesicles, ectosomes, and large oncosomes (Nakano, Garnier, & Rak, 2015)

In essence, vesicle mediated extracellular communication occurs when a specific cell types generates a molecule, such as Otx2, packages the molecule into a vesicle, and releases the vesicle out of the cell. The vesicle can travel through blood, cerebral spinal fluid, or interstitial fluid to its target cell. The advantage of this packaging system is that vesicles are stable structures and resistant to degradation, thus they can travel relatively long distances (Gyorgy, et al., 2011)

Because we identified Otx2 in the choroid plexus, in vesicles in the cerebral spinal fluid, and concentrated in the amygdala near the lateral ventricle, we believe that the protein Otx2 is synthesized in the choroid plexus, packaged into extracellular vesicles, and released into the cerebral spinal fluid. After release into the cerebral spinal fluid, vesicles travel through the cerebral spinal fluid until they reach the amygdala. Once the vesicles enter the amygdala they release molecules of Otx2 that bind to perineuronal nets surrounding PV+ GABAergic interneurons.

We hypothesized that amygdala samples from individuals with schizophrenia would have fewer vesicles filled with Otx2 compared to samples from healthy control individuals. In individuals with schizophrenia a decrease in the amount of vesicles filled with Otx2 could result in a deficit in the amount of Otx2 available for perineuronal nets and PV+ GABAergic interneurons. A deficit in available Otx2 may result in impaired

perineuronal nets, which could lead to a disruption in PV+ GABAergic interneuron function as seen in schizophrenia.

However, quantification and comparison of vesicle number between samples from individuals with and without schizophrenia revealed that there was not a significant difference in the number of vesicle between the two groups.

However, during the process of vesicle quantification, it was observed that vesicles that contained Otx2 were heterogeneous in size and color. This indicated that vesicles did not contain the same amount of Otx2. Therefore, the amount of Otx2 protein in vesicles was measured using an enzyme linked immunosorbent assay (ELISA).

ELISA assays are considered to be relatively rapid procedures, often used in clinical settings to screen for various diseases such as Lyme disease, rheumatoid arthritis, and West Nile virus (Lequin, 2005). If the molecular underpinnings of schizophrenia were elucidated it may one day be feasible to use an ELISA assay to detect the condition of schizophrenia in symptomatic individuals.

ELISA experiments carried out for this thesis project showed a decrease in the amount Otx2 in postmortem samples donated by individuals with schizophrenia. This indicates that although the number of vesicles that carry Otx2 is the same between samples donated by individuals with schizophrenia and healthy control subjects, the quantity of Otx2 protein inside the vesicle may be decreased in samples donated by individuals with schizophrenia.

In this case the hypothesis that a decrease in Otx2 contributes to the pathophysiology of schizophrenia by disruption in GABAergic transmission is supported by the ELISA findings. Optimistically, this indicates that a potential ELISA based

screening assay for schizophrenia could be developed based on measurement of Otx2 concentration in cerebral spinal fluid samples.

Future experimental directions should test additional cerebral spinal fluid samples to confirm these reported preliminary findings. In addition, ELISA tests for Otx2 in blood serum samples should be carried out because in a clinical setting a blood test is a less invasive procedure compared to a spinal tap.

In conclusion, although we hypothesized that there would be fewer vesicles that contained Otx2 in postmortem amygdala samples donated by individuals diagnosed with schizophrenia; this post mortem study did not support this hypothesis. However, when vesicles that contained Otx2 in the amygdala were counted it was observed that the vesicles were heterogeneous with respect to color and size. This observation indicated that there could be differences in the amount of Otx2 contained in each vesicle.

Therefore, an ELISA assay was performed and revealed that average Otx2 levels in cerebral spinal fluid samples were decreased in post mortem samples donated by individuals with schizophrenia. Ideally, in the future, it will be possible to identify individuals with schizophrenia by their unique genetic make-up and drugs tailored to those unique genetic characteristics will be developed. The consideration of Otx2 as a potential biomarker for the condition of schizophrenia is a preliminary step in this endeavor.

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